

# **Transglutaminase 2 Null Macrophages Respond to Lipopolysaccharide Stimulation by Elevated Proinflammatory Cytokine Production due to an Enhanced $\alpha_v\beta_3$ Integrin-induced Src Tyrosine Kinase Signaling**

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**Abbreviations used in this paper:**

I $\kappa$ B $\alpha$ , inhibitory subunit of NF kappa B alpha

LPS, lipopolysaccharide

NF- $\kappa$ B, nuclear factor-kappaB

PP2, Src tyrosine kinase family inhibitor

TG2, transglutaminase 2

TG2-X, crosslinking activity mutant TG2

TG2-G1-G2, guanine nucleotide binding mutant forms of TG2

TG2-S, secretion mutant TG2

TLR4, toll-like receptor 4

VN, vitronectin

## Abstract

Transglutaminase 2 (TG2) is a protein crosslinking enzyme with several additional biochemical functions. Loss of TG2 *in vivo* results in impaired phagocytosis of apoptotic cells and altered proinflammatory cytokine production by macrophages engulfing apoptotic cells leading to autoimmunity. It has been proposed that TG2 acts as an integrin  $\beta_3$  coreceptor in the engulfment process, while altered proinflammatory cytokine production is related to the lack of latent TGF $\beta$  activation by TG2 null macrophages. Here we report that TG2 null macrophages respond to lipopolysaccharide treatment by elevated IL-6 and TNF $\alpha$  production. Though TGF $\beta$  has been proposed to act as a feed back regulator of proinflammatory cytokine production in LPS-stimulated macrophages, this phenomenon is not related to the lack of active TGF $\beta$  production. Instead, in the absence of TG2 integrin  $\beta_3$  maintains an elevated basal Src family kinase activity in macrophages, which leads to enhanced phosphorylation and degradation of the I $\kappa$ B $\alpha$ . Low basal levels of I $\kappa$ B $\alpha$  explain the enhanced sensitivity of TG2 null macrophages to signals that regulate NF- $\kappa$ B. Our data suggest that TG2 null macrophages bear a proinflammatory phenotype, which might contribute to the enhanced susceptibility of these mice to develop autoimmunity and atherosclerosis.

Key words: inflammation, macrophages, Toll like receptors, NF- $\kappa$ B pathway, TGF-beta

# 1. Introduction

Transglutaminases are a family of thiol- and  $\text{Ca}^{2+}$ -dependent acyl transferases that catalyze the formation of a covalent bond between the  $\gamma$ -carboxamide groups of peptide-bound glutamine residues and various primary amines, including the  $\epsilon$ -amino group of lysine in target proteins [1]. Eight distinct enzymatically active transglutaminases have so far been described [2]. TG2 is very unique among the TG family members, because in addition to catalyzing the formation of protein crosslinks, it also possesses GTPase, protein disulfide isomerase and protein kinase activities [3]. Although TG2 is localized predominantly in the cytoplasm, substantial amounts of the protein is present also on the surface in various cell types and in the extracellular matrix [4,5], despite of the fact that TG2 has no leader sequence, hydrophobic domains or posttranslational modifications for targeting the endoplasmic reticulum or Golgi apparatus. In addition, TG2 also interacts with integrins of the  $\beta_1$  and  $\beta_3$  subfamilies, and integrin/TG2 complexes are detected inside the cell during biosynthesis and accumulate as co-receptors on the cell surface [6,7].

We have previously reported that TG2<sup>-/-</sup> mice develop an age-dependent autoimmunity due to defective *in vivo* clearance of apoptotic cells [8]. TG2 expressed on the cell surface of macrophages promotes the engulfment of apoptotic cells by forming a complex with both integrin  $\beta_3$  and its bridging molecule, milk fat globule-EGF factor 8 [9]. In the absence of TG2 integrin  $\beta_3$  does not accumulate properly in the phagocytic cup, and the apoptotic cell-induced activation of Rac1 is impaired. As a compensatory response, TG2 null macrophages elevate the expression levels of both integrin  $\beta_3$  and RhoG, and the cell adhesion-induced integrin  $\beta_3$  signaling is enhanced [9,10].

Though studies on knock out mice, in which the *in vivo* clearance of apoptotic cells is impaired, suggested that impaired phagocytosis of apoptotic cells might lead to the development of autoimmunity [11, 12], it is very likely that altered proinflammatory cytokine

production by macrophages engulfing apoptotic cells contributes to the phenomenon, since CD14<sup>-/-</sup> mice, which show clear defect in the *in vivo* clearance of apoptotic cells, but no alteration in proinflammatory cytokine production during engulfment of apoptotic cells, do not develop autoimmunity [13]. In contrast, macrophages from TG2, Mer tyrosine kinase or complement C1q null mice, which show also a defect in the *in vivo* clearance of apoptotic cells, but develop autoimmunity, produce elevated levels of proinflammatory cytokines, when engulf apoptotic cells [14-16]. Since TGF- $\beta$  was shown to contribute to the down-regulation of proinflammatory cytokine production by macrophages engulfing apoptotic cells [17], and TG2 is required for the activation of latent TGF $\beta$  produced by macrophages [18], it has been suggested that altered proinflammatory cytokine production by TG2 null macrophages is related to the lack of TGF- $\beta$  activation [14]. Since TGF- $\beta$  has been proposed to act also as an autocrine feed back regulator of proinflammatory cytokine production of LPS-stimulated macrophages [19], we decided to investigate whether loss of TG2 could also alter LPS-induced production of proinflammatory cytokines.

The innate immune system copes with infection by producing proinflammatory mediators such as TNF- $\alpha$  and IL-6. Conserved pathogen-associated molecular patterns on microorganisms are recognized by Toll-like receptors, which mediate signals to activate immune cells via association with different intracellular adaptor proteins [20]. LPS, a component of the cell wall of Gram-negative bacteria, is recognized by TLR4 together with accessory molecules such as CD14, and TLR4 then transduces LPS signaling via both myeloid differentiation factor 88-dependent and -independent pathways, each of which activates NF- $\kappa$ B, a transcription factor, that controls the expression of various proinflammatory cytokine genes [21]. Among many others, it has been reported that  $\alpha_v\beta_3$  integrin signaling can also lead to NF- $\kappa$ B activation and enhance LPS-induced NF $\kappa$ B signaling [22,23]. The pathway involves the Src tyrosine kinase, which phosphorylates and

activates inhibitory kappa kinase beta. Inhibitory kappa kinase beta then promotes the degradation of I $\kappa$ B $\alpha$ , a negative regulator of NF- $\kappa$ B activation [24].

Here we report that TG2 null macrophages indeed respond by elevated proinflammatory cytokine production to LPS stimulation, however, the alteration is not related to the lack of TGF- $\beta$  activation, but to an enhanced  $\alpha_v\beta_3$  integrin signaling, which maintains a lower basal I $\kappa$ B $\alpha$  level.

## 2. Materials and Methods

### 2.1 Cell culture

Resident peritoneal macrophages were isolated from 3-6 months old C57B/6 or TG2<sup>-/-</sup> mice [45] by peritoneal lavage after being killed by ether anesthesia. Study protocols were approved by the Animal Care Committee of the University of Debrecen. Peritoneal macrophages were maintained for two days prior to experiments in RPMI1640 medium supplemented with 10% FBS.

### 2.2 Adenoviral gene delivery system

Recombinant, replication-deficient adenoviral vectors encoding either LacZ and the murine TG2 gene or the secretion deficient (TG2-S), guanine nucleotide binding deficient (TG2-G1 and TG2-G2) or crosslinking function deficient (TG2-X) TG2 mutants were produced using the AdEasy XL system (Stratagene) according to the manufacturer's instruction. Virus titers were determined by plaque assay in 293 cells after exposing them to virus for 48 hours in DMEM medium supplemented with 2% serum and antibiotics. For gene delivery,  $2 \times 10^6$  macrophages were exposed to  $2 \times 10^9$  PFU/ml virus particles for 48 hrs in the same medium. LacZ expression was determined with X-gal staining, while TG2 expression by Western blot analysis using anti-TG2 specific antibodies.

### 2.3 Determination of cytokine production

Wild-type and TG2 null peritoneal macrophages were seeded onto 24-well plates at a density of  $5 \times 10^5$  cells/well in 500  $\mu$ l medium. Cells were treated with 100 ng/ml crude LPS (Sigma) for one hour in the presence or absence of increasing concentrations of recombinant TGF $\beta$  (Serotec) as indicated in the results section. After one hour LPS was removed and fresh

medium was added to the cells containing either recombinant TGF $\beta$ , 4 ng/ml neutralizing anti-pan TGF $\beta$  antibody (R&D Systems), isotype control antibody or vehicle. Supernatants were collected and frozen at the indicated time points. IL-6, TNF $\alpha$  and active TGF $\beta$  cytokine levels were measured with R&D Systems ELISA kits.

## 2.4 Western blot

Wild-type and TG2 null peritoneal macrophages were seeded onto 6-well plates at a density of  $2 \times 10^6$  cells/well. Cells were treated with 100 ng/ml LPS for the indicated time periods, pretreated with 1  $\mu$ g/ml soluble vitronectin (Sigma) for 30 minutes or pretreated with 2  $\mu$ M PP2 (Calbiochem) for 24 hours. Cells were harvested at the indicated time points and boiled 2x sample buffer and loaded onto SDS PAGE gels. PVDF membranes were probed with anti-I $\kappa$ B $\alpha$  (Santa Cruz Biotechnology), anti-cSrc (Santa Cruz Biotechnology), anti-phospho(Tyr416)-Src (Cells Signaling Technology), anti-integrin  $\beta$ 3 (MBL International), anti-phospho (Tyr474) integrin  $\beta$ 3 ( Santa Cruz Biotechnology ), anti-TG2 (Santa Cruz Biotechnology) and  $\beta$ -actin antibodies (Sigma).

## 2.5 Determination of NF- $\kappa$ B p65 nuclear translocation

$10^7$  wild-type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for 30, 60 and 120 minutes. Cell were rinsed with ice cold PBS and nuclei were isolated with Nuclei EZ kit (Sigma) according to manufacturer's instruction. Nuclear p65 subunit was detected with TransAM p65 kit (ActiveMotif) according to manufacturer's instruction.

## 2.6 Flow cytometry



$5 \times 10^5$  peritoneal macrophages were labeled in 50  $\mu$ l PBS with FITC conjugated anti-CD14 antibody (Pharmingen) or rabbit-anti mouse TLR4 antibody (Santa Cruz Biotechnology) washed with PBS and incubated further with FITC-anti-rabbit antibody. Cells were analyzed on a Becton Dickinson FACS Calibur platform.

## 2.7 Quantitative PCR

After various treatments  $2 \times 10^6$  peritoneal macrophages were washed with ice-cold PBS. RNA was extracted with Tri-reagent. cDNA was synthesized with High-Capacity cDNA Archive Kit (Applied Biosystems) according to manufacturers instruction. Cyclophilin D, IL-6 and TNF $\alpha$  levels were determined with Taq-Man PCR using FAM-MGB labeled probes (Applied Biosystems) on ABI7900 platform. Gene expression was normalized to cyclophilin D expression.

## 2.8 mRNA stability determination

Wild-type and TG2 null peritoneal macrophages were pre-treated with 100 ng/ml LPS for 1 h followed by addition of 1  $\mu$ g/ml Actinomycin D (Sigma). Total RNA was isolated at the indicated time points and TNF $\alpha$  mRNA was measured by quantitative RT-PCR.

### 3. Results

#### 3.1 TG2 null macrophages respond by elevated proinflammatory cytokine production to LPS treatment

To determine the time course of LPS-stimulated proinflammatory cytokine production of wild type resident peritoneal macrophages, macrophages were exposed to 100 ng/ml LPS, and the LPS-induced IL-6 and TNF $\alpha$  production was determined by ELISA at various time points (Fig.1). To avoid detection of *in vivo* induced proinflammatory cytokine production, macrophages were plated for 2 days, by a time when no more endogenous cytokine production was found, before addition of the LPS. As shown in Figure 1, the IL-6 and TNF $\alpha$  production of wild-type peritoneal macrophages reached their maximum at 6h following LPS stimulation, and these levels remained with no significant alterations during the following 20 hours (data not shown). While IL-6 production started to raise only after one hour of LPS stimulation (Fig. 1A), a significant amount of TNF $\alpha$  was detected already at one hour of LPS stimulation (Fig. 1B) indicating that some TNF $\alpha$  is stored within the macrophages, which is released upon LPS-stimulation. Peritoneal macrophages responded with more IL-6 than TNF $\alpha$  production to LPS stimulation.

Loss of TG2 did not affect the kinetics of IL-6 or TNF $\alpha$  production, but the levels of proinflammatory cytokines produced by TG2 null macrophages were higher at each time points as compared to that produced by the wild-type ones. These data indicate that in the absence of TG2 macrophages are more sensitive to LPS stimulation than their wild type counterparts.

#### 3.2 Elevated proinflammatory cytokine production by TG2 null macrophages is not related to the lack of TGF $\beta$ activation

Since TG2 is required for the activation of latent TGF $\beta$  produced by macrophages [18], and active TGF $\beta$  has been proposed to act as an autocrine feed back regulator of proinflammatory cytokine production of LPS-stimulated macrophages [19], we decided to test whether the enhanced proinflammatory cytokine production is related to the lack of TGF $\beta$  activation by TG2 null macrophages.

As shown in Figure 2A, in line with the previously reported data about the requirement of TG2 for the activation of latent TGF $\beta$  [18], TG2 null macrophages indeed were unable to produce detectable amounts of active TGF $\beta$ . However, a pan TGF $\beta$  neutralizing antibody [25] failed to enhance the LPS-induced early proinflammatory cytokine production (Fig.2B). The neutralising antibody concentration used was sufficient to block all the active TGF $\beta$ , because after addition of the neutralising antibody no active TGF $\beta$  was detectable in the supernatants by ELISA (data not shown). In addition, recombinant TGF $\beta$ , in the concentrations we detected active TGF $\beta$  in the cell culture medium, could not significantly affect LPS-induced proinflammatory cytokine production of TG2 null macrophages (Fig. 2C), indicating that TGF $\beta$  has no effect on the short-term LPS-induced cytokine formation of macrophages, and not the lack TGF $\beta$  production is responsible for the enhanced proinflammatory cytokine formation by TG2 null macrophages. So we decided to test further the characteristics of LPS signaling in TG2 null peritoneal macrophages.

### 3.3 Basal levels of I $\kappa$ B $\alpha$ are decreased in TG2 null macrophages

LPS is recognized by macrophages via TLR4 together with the accessory molecule CD14 [21]. To test, whether the expression of the LPS sensing receptors have changed in TG2 null macrophages, the cell surface expression levels of CD14 and TLR4 were detected by flow cytometry. However, as shown in Fig. 3A, no change in the cell surface expression of these

receptors was found in TG2 null macrophages, indicating that not an altered expression of these receptors is responsible for the enhanced LPS sensitivity.

There are several levels, at which LPS-induced production of  $\text{TNF}\alpha$  and IL-6 can be controlled. First we decided to test whether their transcription is altered. As shown in Figure 3B, using Q-PCR technique we could not detect basal levels of mRNA for the two proinflammatory cytokines. Exposure to LPS enhanced the expression of  $\text{TNF}\alpha$  and IL6 in both types of macrophages, but the TG2 null macrophages showed about 2 and 4 fold higher mRNA productions, respectively. To differentiate whether the transcription or the stability of the mRNA was altered in the absence of TG2, LPS-stimulated macrophages were exposed to actinomycin D, a transcription inhibitor and the time dependent decrease in the mRNA expression of  $\text{TNF}\alpha$  was followed in both wild-type and TG2 null macrophages (Fig.3C). Since no change in the kinetics of the mRNA degradation was found, it is likely that the loss of TG2 alters the proinflammatory cytokine production at transcriptional level.

Since the signaling pathways induced by LPS transduce their effect on proinflammatory cytokine production partly via activating NF- $\kappa$ B, and these pathways regulate the degradation of the inhibitory subunit  $\text{I}\kappa\text{B}\alpha$  [21], a negative regulator of NF- $\kappa$ B, we decided to determine the  $\text{I}\kappa\text{B}\alpha$  levels in wild-type and TG2 null macrophages following LPS stimulation. As shown in Figure 3D, there was no change in the kinetics of the  $\text{I}\kappa\text{B}\alpha$  degradation induced by LPS stimulation, but the basal levels of  $\text{I}\kappa\text{B}\alpha$  in TG2 null macrophages were significantly lower than that of the wild type cells.

$\text{I}\kappa\text{B}\alpha$  is commonly associated with the NF- $\kappa$ B dimer  $\text{p50(NF-}\kappa\text{B1)/p65(RelA)}$ . Following proteolytic degradation of  $\text{I}\kappa\text{B}\alpha$  by the proteasome, the NF- $\kappa$ B dimer becomes free to enter the nucleus and to activate transcription of target genes. While NF- $\kappa$ B p65 is transcriptionally active, NF- $\kappa$ B p50 does not possess a transactivation domain [24]. Thus, though p65 and p50 can synergistically activate for example the  $\text{TNF}\alpha$  promoter [26], the

presence of p65 is crucial for the initiation of transcription [24]. That is why we decided to test the nuclear translocation of the p65 subunit of NF- $\kappa$ B by the TransAM p65 kit (ActiveMotif). As shown in Figure 3E, LPS stimulation induced the nuclear translocation of p65 in both types of macrophages. However, in accordance with the lower I $\kappa$ B $\alpha$  levels in TG2 null cells, both the basal and the LPS-induced levels of p65 were higher in the nucleus of TG2 null macrophages than in that of the wild-type macrophages at each time point tested. These data indicate that the loss of TG2 alters a signaling pathway that is coupled to the control of I $\kappa$ B $\alpha$  levels.

### 3.4 TG2 is required on the cell surface to decrease LPS-induced proinflammatory cytokine production in TG2 null macrophages

Previous studies have already shown association between NF- $\kappa$ B activation and TG2. In LPS-stimulated microglial cells TG2 activates NF- $\kappa$ B via a novel pathway. Rather than stimulating phosphorylation and degradation of I $\kappa$ B $\alpha$ , TG2 interferes with its action by protein polymerization [27]. On the other hand, in cancer cells overexpression of TG2 enhances NF- $\kappa$ B activation by promoting integrin signaling [28]. However, if these mechanisms exist in macrophages, loss of TG2 should lead to a decreased, not to an enhanced LPS signaling.

To answer which biological functions of TG2 are required to downregulate LPS-induced proinflammatory cytokine production, adenoviral gene delivery system was used to transfect primary peritoneal macrophages with various mutants of TG2 (Fig. 4A). The following TG2 mutants were tested: a crosslinking activity mutant (TG2-X) by replacement of catalytic Cys<sup>277</sup> by Ser [29], two guanine nucleotide binding mutants by replacement of Lys<sup>172</sup> and Phe<sup>173</sup> by Asn and Asp (TG2-G1) [30], and of Glu<sup>578</sup> and Arg<sup>579</sup> by Gln and Glu (TG2-G2) [31], and a secretion mutant (TG2-S) by replacement of Tyr<sup>274</sup> by Ala [32]. As shown in Figure 4B, using IL-6 production as a read out, only the wild type and the

crosslinking mutant were able to fully revert the LPS sensitive phenotype, while the secretion mutant and the two guanine nucleotide binding mutants were ineffective. Our data indicate that cell surface TG2 regulates negatively the LPS-induced proinflammatory cytokine production, and the crosslinking activity is not required for this effect. In addition, these data confirm those findings, which suggested that the enhanced LPS-induced proinflammatory cytokine production of TG2 null macrophages is not related to the lack of TGF $\beta$  activation, as it would require the crosslinking activity of TG2 [18].

### 3.5 Altered $\alpha_v\beta_3$ signaling is responsible for the enhanced LPS-induced proinflammatory cytokine production in macrophages

On the cell surface TG2 has been shown to act as an integrin-binding adhesion coreceptor and acting so to suppress Src kinase activity [33]. Since it has been reported that  $\alpha_v\beta_3$  integrin signaling can lead to NF- $\kappa$ B activation and enhance LPS-induced NF- $\kappa$ B signaling via activating Src kinase [22,23], we decided to test the potential role of an altered  $\alpha_v\beta_3$  integrin signaling in the enhanced LPS-induced proinflammatory cytokine production of TG2 null macrophages. Preincubation of TG2 null macrophages with soluble vitronectin, an inhibitor of the  $\alpha_v\beta_3$  integrin signaling [34,35], decreased the LPS-induced pro-inflammatory cytokine production on mRNA levels, indicating that  $\alpha_v\beta_3$  integrin signaling promotes the LPS-induced proinflammatory cytokine production in TG2 null macrophages (Fig. 4C). Interestingly, the same treatment enhanced the proinflammatory cytokine production by wild-type macrophages (Fig. 4D). In line with these observations, LPS-induced I $\kappa$ B $\alpha$  levels were further decreased following vitronectin treatment in wild-type cells (Fig. 4F), but remained more elevated in knock out cells (Fig. 4E). Since cell surface TG2 was reported to crosslink soluble vitronectin [36], and crosslinked soluble vitronectin might enhance instead of

inhibiting  $\alpha_v\beta_3$  integrin signaling, for wild-type macrophages we repeated the experiments in the presence of R294, a non-permeable TG2 inhibitor, which blocks the crosslinking activity of cell surface TG2 [compound 4 in 37]. As shown in Figure 4D, in the presence of the TG2 inhibitor and soluble vitronectin wild-type cells responded to LPS with nearly the same amount of cytokine mRNA expression as in the absence of them. In control experiments addition of the TG2 inhibitor did not influence the response of TG2 null cells to soluble vitronectin (Fig. 4C). Alterations in the I $\kappa$ B levels mirrored these changes in the cytokine mRNA expression (Fig. 4E and F). Altogether these data indicate that under our experimental conditions in wild type cells ligand-activated  $\alpha_v\beta_3$  integrin signaling, which can be inhibited by soluble vitronectin, does not play a determining role in influencing LPS signaling (though stimulation of it by crosslinked vitonectin is capable of its enhancement), while in TG2 null cells it does.

### 3.6 In TG2 null macrophages enhanced $\alpha_v\beta_3$ integrin-induced src family tyrosine kinase activation is responsible for the enhanced NF $\kappa$ B signaling

Next we decided to test the activation state of Src family tyrosine kinases in TG2 null macrophages. The Src-family tyrosine kinases are highly conserved allosteric enzymes playing a key role in integrin cellular signaling. Phosphorylation of Tyr416 plays a central role in their activation [38]. In line with the report, which suggested that TG2 might negatively control  $\alpha_v\beta_3$  integrin-regulated Src kinase activity [33], an enhanced phosphorylation of c-src family kinases was detected at Tyr 416 in TG2 null cells without a detectable change in the c-src protein levels as compared to the wild type cells (Fig.5A). LPS stimulation enhanced the amount of phosphorylated Src family tyrosine kinases in both types of macrophages (Fig. 5A), in line with previous publications, which showed that Src kinase is also involved in LPS signaling [23, 39].

The cytoplasmic domain of  $\beta_3$  integrin contains tyrosines at positions 747 and 759 in domains that have been implicated in regulation of  $\alpha_v\beta_3$  function and that serve as potential substrates for Src family kinases [40]. Phosphorylation of Tyr at residue 747 was reported to be required for optimal post-ligand binding effects [41], as well as for proper binding of the integrin ligands [40] thus participating in both in the “outside in” and “inside out” integrin signaling. To test the activation state of  $\beta_3$  integrin in TG2 null cells both the level of  $\beta_3$  integrin and the phosphorylation state of its Tyr747 residue were determined by Western blot analysis. As shown in Figure 5B, in accordance with our previous findings [9,10] the levels of  $\beta_3$  integrin were elevated in TG2 null macrophages. While in wild-type cells  $\beta_3$  integrin was only slightly phosphorylated at the Tyr747 site, in TG2 null cells the phosphorylation level was much more pronounced indicating an enhanced activation of integrin  $\beta_3$  in the absence of TG2. However, phosphorylation of  $\beta_3$  integrin and Src tyrosine kinases seems to be related to each other in reciprocal way, as inhibition of  $\alpha_v\beta_3$  integrin by soluble vitronectin decreased the phosphorylation level of Src tyrosine kinase, and similarly inhibition of Src tyrosine kinase with 2  $\mu$ M PP2 [(4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine)], an Src tyrosine kinase family inhibitor, inhibited the phosphorylation state of  $\alpha_v\beta_3$  integrin (Fig. 5C).

Then we tested whether the decreased I $\kappa$ B $\alpha$  levels could be related to the enhanced activation of Src. Preincubation of macrophages with 2  $\mu$ M PP2 for 24 hours did not affect their viability, but as shown in Figure 5D, equalized the basal levels of I $\kappa$ B $\alpha$  detected in the wild-type and TG2 null macrophages indicating that the enhanced activity of Src kinase is responsible for the altered I $\kappa$ B $\alpha$  levels in TG2 null cells. In addition, inhibition of Src kinase delayed LPS-induced degradation of I $\kappa$ B $\alpha$  in both types of cells, and in the presence of PP2 no difference was found in the I $\kappa$ B $\alpha$  levels following addition of LPS. Finally, addition of



PP2 decreased, but at the same equalized the LPS-induced mRNA production of the two proinflammatory cytokines within the wild-type and TG2 null macrophages (Fig. 4C and D). All together these data provide evidence for the involvement of the  $\alpha_v\beta_3$  integrin regulated Src family tyrosine kinases in the altered LPS signaling in TG2 null macrophages.

## 4. Discussion

Previous studies have shown that TG2 null mice develop an age-dependent autoimmunity due to defective *in vivo* clearance of apoptotic cells by macrophages [8]. In addition, it was also demonstrated that TG2 null macrophages unlike their wild-type counterparts, when are exposed to apoptotic cells, release proinflammatory cytokines, including IL-6 [14]. In the present study the LPS responsiveness of TG2 null macrophages was investigated. We found that in the absence of TG2 macrophages become more sensitive to LPS treatment and respond by enhanced proinflammatory cytokine production as compared to their wild-type counterparts.

Increasing evidence suggests that TGF $\beta$  released by macrophages exposed to either LPS [18] or apoptotic cells [17] play a key role in the control or termination of the proinflammatory response. However, to act so macrophages have to be exposed prior or for a longer time period to the endogenous or rTGF $\beta$  [42]. As a result, though we confirmed previous suggestions that TG2 is required for TGF $\beta$  activation by murine macrophages [18], testing by addition of neutralizing anti-TGF $\beta$  antibodies, lack of active TGF $\beta$  production did not significantly affect the LPS-induced proinflammatory cytokine production of TG2 null macrophages in short term cultures.

Instead we found that loss of TG2 altered the  $\alpha_v\beta_3$  integrin signaling in macrophages leading to an enhanced basal Src tyrosine kinase activity. The crosslinking activity of TG2

was not required for proper  $\alpha_v\beta_3$  integrin signaling and LPS-induced cytokine production, but TG2 had to be expressed on the cell surface and bind guanine nucleotides. These results are in agreement with previous findings, which demonstrated that TG2 modifies integrin signaling in guanine nucleotide bound form [43]. Though TG2 can act as a G protein in many physiological settings, in the context of regulating integrin signaling proper guanine nucleotide binding of TG2 was suggested to be required for stabilizing the protein in a conformation state that can facilitate physical interactions with other proteins, such as integrins [43]. Our findings seem to confirm that of others, which showed a synergism between  $\alpha_v\beta_3$  integrin signaling and LPS sensitivity [23].

Interestingly, though loss of TG2 sensitized macrophages to LPS, loss of TG2 prevented mice from the endotoxic shock induced by LPS [44]. The pathogenesis of the endotoxic shock, however, is very complex, and the various effects of the multifunctional protein TG2 in various tissues, such as heart, kidney or neutrophils, explain the controversy between our findings and the *in vivo* results [44]. Our data, however, demonstrate that TG2 null macrophages might be more sensitive to all stimuli that lead to proinflammatory cytokine production via activation of the NF- $\kappa$ B pathway. This proinflammatory phenotype of TG2 null macrophages might contribute to the development of autoimmunity in these mice [8] and their increased sensitivity to develop atherosclerosis [45, 46].

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## References

- [1] Greenberg, C.D., Birckbichler, P.J. and Rice, R.H. Transglutaminases: multifunctional cross-linking enzymes that stabilize tissues, *FASEB J.* 5 (1991) 3071-3077.
- [2] Lorand, L., and Graham, R.M. Transglutaminases: crosslinking enzymes with pleiotropic functions, *Nat. Rev. Mol. Cell. Biol.* 4 (2003)140-156.
- [3] Fésüs, L., and Piacentini, M. Transglutaminase 2: an enigmatic enzyme with diverse functions, *Trends Biochem. Sci.* . 27 (2002) 534-539.
- [4] Thomázy, V., and Fésüs, L. Differential expression of tissue transglutaminase in human cells. An immunohistochemical study, *Cell Tissue Res.* 255 (1989) 215-224.
- [5] Upchurch, H.F., Conway, E., Patterson, M.K. and Maxwell, M.D. Localization of cellular transglutaminase on the extracellular matrix after wounding: characteristics of the matrix bound enzyme, *J. Cell Physiol.* 149 (1991) 375-382.
- [6] Akimov, S.S., Krylov, D., Fleischman, L.F. and Belkin, A. Tissue transglutaminase is an integrin-binding adhesion coreceptor for fibronectin, *J. Cell. Bio.* 148 (2000) 825-838.
- [7] Akimov, S.S., and Belkin, A.M. Cell surface tissue transglutaminase is involved in adhesion and migration of monocytic cells on fibronectin, *Blood* 98 (2001) 1567-1576.
- [8] Szondy, Z., Sarang, S., Molnár, P., Németh, T., Piacentini, M., Mastroberardino, P.G., Falasca, L., Aeschlimann, D., Szegezdi, E., Lakos, G., Rajnavölgyi, E., Birckbichler, P.J., Melino, G. and Fésüs, L. TGase2<sup>-/-</sup> mice reveal a phagocytosis-associated crosstalk between macrophages and apoptotic cells, *Proc. Natl. Acad. Sci. US.* 100 (2003) 7812-7817.

- [9] Tóth, B., Garabuczi, E., Sarang, Z., Vereb, G., Vámosi, G., Aeschlimann, D., Blaskó, B., Bécsi, B., Erdődi, F., Lacy-Hulbert, A., Zhang, A., Falasca, L., Birge, R.B., Balajthy, Z., Melino, G., Fésüs, L. and Szondy, Z. Transglutaminase 2 is needed for the formation of an efficient phagocyte portal in macrophages engulfing apoptotic cells, *J. Immuno.* 182 (2009) 2084-2092.
- [10] Tóth, B., Sarang, Z., Vereb, G., Zhang, A., Tanaka, S., Melino, G., Fésüs, L., Szondy, Z. Over-expression of integrin beta3 can partially overcome the defect of integrin beta3 signaling in transglutaminase 2 null macrophages, *Immunol. Lett.* 36 (2009) 625-631.
- [11] Savill, J., Dransfield, I., Gregory, C. and Haslett, C. A blast from the past: clearance of apoptotic cells regulates immune responses, *Nat. Rev. Immunol.* 2 (2002) 965-975.
- [12] Tanaka, M., and Miyake, Y. Apoptotic cell clearance and autoimmune disorder, *Curr. Med. Chem.* 14 (2007) 2892-7.
- [13] Devitt, A., Parker, K.G., Ogden, C.A., Oldreive, C., Clay, M.F., Melville, L.A., Bellamy, C.O., Lacy-Hulbert, A., Gangloff, S.C., Goyert, S.M. and Gregory, C.D. Persistence of apoptotic cells without autoimmune disease or inflammation in CD14<sup>-/-</sup> mice, *J. Cell. Biol.* 167 (2004) 1161-70.
- [14] Falasca, L., Iadevaia, V., Ciccocanti, F., Melino, G., Serafino, A. and Piacentini, M. Transglutaminase type II is a key element in the regulation of the anti-inflammatory response elicited by apoptotic cell engulfment, *J. Immunol.* 174 (2005) 7330-7340.
- [15] Tibrewal, N., Wu, Y., D'mello, V., Akakura, R., George, T.C., Varnum, B. and Birge, R.B. Autophosphorylation docking site Tyr-867 in Mer receptor tyrosine kinase allows for dissociation of multiple signaling pathways for phagocytosis of apoptotic cells and

- down-modulation of lipopolysaccharide-inducible NF-kappaB transcriptional activation, *J. Biol. Chem.* 283 (2008) 3618-27.
- [16] Fraser, D.A., Bohlson, S.S., Jasinskiene, N., Rawal, N., Palmarini, G., Ruiz, S., Rochford, R. and Tenner, A.J. C1q and MBL, components of the innate immune system, influence monocyte cytokine expression, *J. Leukoc. Biol.* 80 (2006) 107-116.
- [17] Fadok, V.A., Bratton, D.L., Konowal, A., Freed, P.W., Westcott, J.Y. and Henson, P.M. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF, *J. Clin. Invest.* 101 (1998) 890-898.
- [18] Nunes, I., Shapiro, R.L. and Rifkin, D.B. Characterization of latent TGF-beta activation by murine peritoneal macrophages, *J. Immunol.* 155 (1995) 1450-1459.
- [19] Letterio, J.J., and Roberts, A.B. Regulation of immune responses by TGF-beta, *Annu.Rev. Immunol.* 16 (1998) 137-161.
- [20] O'Neill, L.A., and Bowie, A.G. The family of five: TIR-domain-containing adaptors in Toll-like receptor signalling, *Nat. Rev. Immunol.* 5 (2007) 353-364.
- [21] Kawai, T., and Akira, S. (2006) TLR signaling. *Cell Death Differ.* 13:816-825.
- [22] Courter, D.L., Lomas, L., Scatena, M. and Giachelli, C.M. Src kinase activity is required for integrin alphaVbeta3-mediated activation of nuclear factor-kappaB, *J. Biol. Chem.* 280 (2005) 12145-51.
- [23] Lee, H.S., Moon, C., Lee, H.W., Park, E.M., Cho, M.S. and Kang, J.L. Src tyrosine kinases mediate activations of NF-kappaB and integrin signal during lipopolysaccharide-induced acute lung injury, *J. Immunol.* 179 (2007) 7001-7011.

- [24] Baldwin, A. S. Jr. The NF- $\kappa$ B and I $\kappa$ B proteins: new discoveries and insights, *Annu. Rev. Immunol.* 14 (1996) 649-683.
- [25] Denning, T.L., Wang, Y.C., Patel, S.R., Williams, I.R. and Pulendran, B. Lamina propria macrophages and dendritic cells differentially induce regulatory and interleukin 17-producing T cell responses, *Nat. Immunol.* 8 (2007) 1086-1094.
- [26] Liu, H., Sidiropoulos, P., Song, G., Pagliari, L.J., Birrer, M.J., Stein, B., Anrather, J. and Pope, R.M. TNF- $\alpha$  gene expression in macrophages: Regulation by NF- $\kappa$ B is independent of c-Jun or C/EBP $\beta$ , *J. Immunol.* 164 (2000) 4277-4285.
- [27] Lee, J., Kim, Y.S., Choi, D.H., Bang, M.S., Han, T.R., Joh, T.H. and Kim SY. Transglutaminase 2 induces nuclear factor-kappaB activation via a novel pathway in BV-2 microglia, *J. Biol. Chem.* 279 (2004) 53725-35.
- [28] Verma, A., and Mehta, K. Transglutaminase-mediated activation of nuclear transcription factor-kappaB in cancer cells: a new therapeutic opportunity, *Curr. Cancer Drug Targets* 6 (2007) 559-565.
- [29] Lee, K.N., Arnold, S.A., Birckbichler, P.J., Patterson, M.K., Fraij, B.M., Takeuchi, Y. and Carter, H.A. Site-directed mutagenesis of human tissue transglutaminase: Cys-277 is essential for transglutaminase activity but not for GTPase activity, *Biochim. Biophys. Acta* 1202 (1993) 1-6.
- [30] Iismaa, S.E., Wu, M.J., Nanda, N., Church, W.B. and Graham, R.M. GTP binding and signaling by Gh/transglutaminase II involves distinct residues in a unique GTP-binding pocket, *J. Biol. Chem.* 275 (2000) 18259-18265.

- [31] Begg, G.E., Holman, S.R., Stokes, P.H., Matthews, J.M., Graham, R.M. and Iismaa, S.E. Mutation of a critical arginine in the GTP-binding site of transglutaminase 2 disinhibits intracellular cross-linking activity, *J. Biol. Chem.* 281 (2006) 12603-12609.
- [32] Balklava, Z., Verderio, E., Collighan, R., Gross, S., Adams, J. and Griffin, M. Analysis of tissue transglutaminase function in the migration of Swiss 3T3 fibroblasts: the active-state conformation of the enzyme does not affect cell motility but is important for its secretion, *J. Biol. Chem.* 277 (2002) 16567-16575.
- [33] Janiak, A., Zemskov, E.A. and Belkin, A.M. Cell surface transglutaminase promotes RhoA activation via integrin clustering and suppression of the Src-p190RhoGAP signaling pathway, *Mol. Biol. Cell.* 4 (2006) 1606-19.
- [34] Preissner, K.T. Structure and Biological Role of Vitronectin, *Ann. Rev. Cell. Biol.* 7 (1991) 275-310.
- [35] Savill, J., Dransfield, I., Hogg, N. and Gregory, C. Vitonectin-receptor mediated phagocytosis of cells undergoing apoptosis, *Nature* 343 (1990) 170-173.
- [36] Sane, D.C., Moser, T.L., Pippen, A.M., Parker, C.J., Achyuthan, K.E. and Greenberg, C.S. Vitronectin is a substrate for transglutaminases, *Biochem. Biophys. Res. Commun.* 157 (1988) 115-20.
- [37] Griffin, M., Mongeot, A., Collighan, R., Saint, R.E., Jones, R.A., Coutts, I.G.C. and Rathbone, D.L. Synthesis of potent water-soluble transglutaminase inhibitors, *Bioorg. Med. Chem Lett.* 18 (2008) 5559-5562.
- [38] Banavali, N.K. and Roux, B. Flexibility and charge asymmetry in the activation loop of Src tyrosine kinases, *Proteins.* 74 (2009) 378-389.



- [39] Kang, J.L., Lee, H.W., Kim, H.J., Lee, H.S., Castranova, V., Lim, C.M. and Koh, Y. Inhibition of SRC tyrosine kinases suppresses activation of nuclear factor-kappaB, and serine and tyrosine phosphorylation of IkappaB-alpha in lipopolysaccharide-stimulated raw 264.7 macrophages, *J. Toxicol. Environ. Health* 68 (2005) 1643-1662.
- [40] Datta, A., Huber, F. and Boettiger, D. Phosphorylation of  $\beta_3$  integrin controls ligand binding strength, *J. Biol. Chem.* 277 (2002) 3943-3949.
- [41] Schaffner-Reckinger, E., Gouon, V., Melchior, C., Plançon, S. and Kieffer, N. Distinct involvement of beta3 integrin cytoplasmic domain tyrosine residues 747 and 759 in integrin-mediated cytoskeletal assembly and phosphotyrosine signalling, *J. Biol. Chem.* 273 (1998) 12623-12632.
- [42] Hausmann, E.H., Hao, S.Y., Pace, J.L. and Parmely, M.J. Transforming growth factor beta 1 and gamma interferon provide opposing signals to lipopolysaccharide-activated mouse macrophages, *Infect. Immunol.* 62 (1994) 3625-3632.
- [43] Johnson, K.A., and Terkeltaub, R.A. External GTP-bound transglutaminase 2 is a molecular switch for chondrocyte hypertrophic differentiation and calcification, *J. Biol. Chem.* 280 (2005) 15004-15012.
- [44] Falasca, L., Farrace, M.G., Rinaldi, A., Tuosto, L., Melino, G. and Piacentini, M. Transglutaminase type II is involved in the pathogenesis of endotoxic shock. *J. Immunol.* 180 (2008) 2616-2624.
- [45] Boisvert, W.A., Rose, D.M., Boullier, A., Quehenberger, O., Sydlaske, A., Johnson, K.A., Curtiss, L.K. and Terkeltaub, R. Leukocyte transglutaminase 2 expression limits atherosclerotic lesion size, *Arterioscler Thromb. Vasc. Biol.* 26 (2006) 563-569.

- [46] Van Herck, J.L., Schrijvers, D.M., De Meyer, G.R.Y., Martinet, W., Van Hove, C.E., Bult, H., Vrints, C.J. and Herman AG. Transglutaminase 2 Deficiency Decreases Plaque Fibrosis and Increases Plaque Inflammation in Apolipoprotein-E-Deficient Mice, *J. Vasc. Res.* 47 (2010) 231-240.
- [47] De Laurenzi, V. and Melino, G. Gene disruption of tissue transglutaminase, *Mol. Cell. Biol.* 21 (2001) 148-155.

## Figure Legends

Figure 1 LPS exposed TG2 null macrophages produce higher amounts of TNF $\alpha$  and IL-6 than wild-type cells. Wild-type (filled circles) and TG2 null (open circles) peritoneal macrophages were treated for 1h with 100 ng/ml LPS. After incubation LPS was washed away and fresh medium was added to the cells. Supernatants were collected at the indicated time points and kept at -20°C until analysis. IL-6 and TNF $\alpha$  cytokine levels were determined by ELISA technique. The results are representative of four independent experiments and are shown as mean  $\pm$  SD. (\*significantly different from wild type,  $p < 0.05$  determined unpaired Student's t-test).

Figure 2 The enhanced proinflammatory cytokine production of LPS-stimulated TG2 null macrophages is not related to the lack of TGF $\beta$  activation. Wild-type (black bars) and TG2 null (grey bars) peritoneal macrophages were treated for 1h with 100 ng/ml LPS (A) alone, or (B) in the presence of 4 ng/ml neutralizing anti-TGF $\beta$  or its isotype control antibody. (C) In addition, TG2 null macrophages were also treated by LPS in the presence of increasing amounts of recombinant TGF $\beta$ . After 1h incubation LPS was washed away, but the indicated compounds were re-added in the fresh medium. Supernatants were collected and frozen after 5h. Active TGF $\beta$ , TNF $\alpha$  and IL-6 cytokine levels were determined by ELISA technique. The results are representative of three independent experiments are shown as mean  $\pm$  SD. (\*significantly different from wild-type,  $p < 0.05$  determined unpaired Student's t-test).

Figure 3 TG2 null macrophages respond to LPS stimulation by an enhanced NF- $\kappa$ B activation as compared to their wild-type counterparts, and this phenomenon is not related to an altered cell surface expression of CD14 or TLR4. (A) Flow cytometric analysis of cell surface CD14

(left) and TLR4 (right) expression of wild-type and TG2 null peritoneal macrophages. Open histograms on the left indicate isotype controls. (B) Quantitative RT-PCR analysis of TNF $\alpha$  and IL6 mRNA expression in wild-type and TG2 null peritoneal macrophages cultured for 1 h with or without 100 ng/ml LPS. The results are representative of three independent experiments and are shown as mean  $\pm$  SD. (C) Measurement of TNF $\alpha$  mRNA stability in wild-type and TG2 null peritoneal macrophages. Cells were treated with 100 ng/ml LPS for 1 h followed by addition of 1  $\mu$ g/ml Actinomycin D. TNF $\alpha$  mRNA was measured by quantitative RT-PCR. (D) Western blot analysis of I $\kappa$ B $\alpha$  degradation in wild-type and TG2 null macrophages after exposure to 100 ng/ml LPS.  $\beta$ -actin was used as a loading control. (E) Determination of the amounts of nuclear p65 NF- $\kappa$ B subunit in control and LPS-stimulated macrophages. Wild-type and TG2 null peritoneal macrophages were treated with 100 ng/ml LPS for the indicated time periods. Nuclear p65 subunit was detected with TransAM p65 kit. The results are representative of three independent experiments and are expressed as fold induction normalized to the wild-type control samples, and are shown as mean  $\pm$  SD. (\*significantly different from wild-type,  $p < 0.05$  determined unpaired Student's t-test).

Figure 4 Integrin  $\beta_3$ -associated cell surface TG2 regulates proinflammatory cytokine production. (A) Western blot analysis showing TG2 expression in TG2 null peritoneal macrophages infected with adenoviruses carrying LacZ gene, wild-type, secretion deficient (TG2-S), guanine nucleotide binding deficient (TG2-G1 and -G2) or crosslinking function deficient (TG2-X) TG2 genes. (B) IL-6 production of wild-type (black bar) or TG2 null (grey bars) peritoneal macrophages infected with the indicated constructs. Macrophages were stimulated with 100 ng/ml LPS for 1 h. After 1h incubation LPS was replaced with fresh medium. Supernatants were collected and frozen after 5h. IL-6 cytokine levels were determined by ELISA technique. Results are shown as mean  $\pm$  SD of three independent

experiments (\* significantly different from LacZ control,  $p < 0.05$  determined by unpaired Student's t-test). (C, D) Quantitative RT-PCR analysis of TNF $\alpha$  and IL6 mRNA expression in (C) TG2 null and (D) wild-type peritoneal macrophages cultured for 1 h with or without 100 ng/ml LPS alone or after one hour pretreatment of soluble vitronectin (VN)(1  $\mu$ g/ml), 2 hrs R294 (100  $\mu$ M) or 24 h pretreatment of PP2 (2  $\mu$ M). Target gene expression was normalized to cyclophilin D. The results are representative of three independent experiments and are expressed as mean  $\pm$  SD. (\* significantly different from the corresponding LPS treated samples,  $p < 0.05$  determined by unpaired Student's t-test). (E, F) Western blot analysis of I $\kappa$ B $\alpha$  degradation in (E) TG2 null and (F) wild-type peritoneal macrophages following treatment by 100 ng/ml LPS alone or together with 1  $\mu$ g/ml VN or 100  $\mu$ M R294 (\* statistically different from LPS treated, \* statistically different,  $p < 0.05$  determined by unpaired Student's t-test).

Figure 5 Loss of TG2 leads to enhanced  $\alpha_v\beta_3$  integrin and Src kinase activity. (A) Increased Src family kinase phosphorylation in TG2 null macrophages. Western blot analysis of Tyr416 phosphorylation of Src family tyrosine kinase in resting wild-type and TG2 null peritoneal macrophages and after 30 min LPS (100 ng/ml) exposure. (B) Increased basal integrin  $\beta_3$  activity in TG2 null macrophages. left: Representative western blot analysis showing Tyr 474 phosphorylation of the integrin  $\beta_3$  subunit in resting wild type and TG2 null peritoneal macrophages.  $\beta$ -actin was used as loading control. right: Densitometric quantification of phospho-integrin  $\beta_3$  level normalized to total integrin  $\beta_3$  levels in wild-type and TG2 null macrophages (n=3, \* statistically different from WT,  $p < 0.05$  determined by unpaired Student's t-test). (C) Effect of 1 hour soluble vitronectin (1  $\mu$ g/ml), pretreatment on the Tyr416 phosphorylation of Src family tyrosine kinase, or 24 h PP2 (2  $\mu$ M) pretreatment on the Tyr 474 phosphorylation of the integrin  $\beta_3$  subunit in resting TG2 null peritoneal

macrophages.  $\beta$ -actin was used as loading control. (D) Western blot analysis of I $\kappa$ B $\alpha$  degradation in wild-type and TG2 null peritoneal macrophages with or without a 24 h PP2 (2  $\mu$ M) pretreatment after triggering or not with 100 ng/ml LPS for 30 min.  $\beta$ -actin was used as loading control (n=3, \* statistically different,  $p < 0.05$  determined by unpaired Student's t-test).

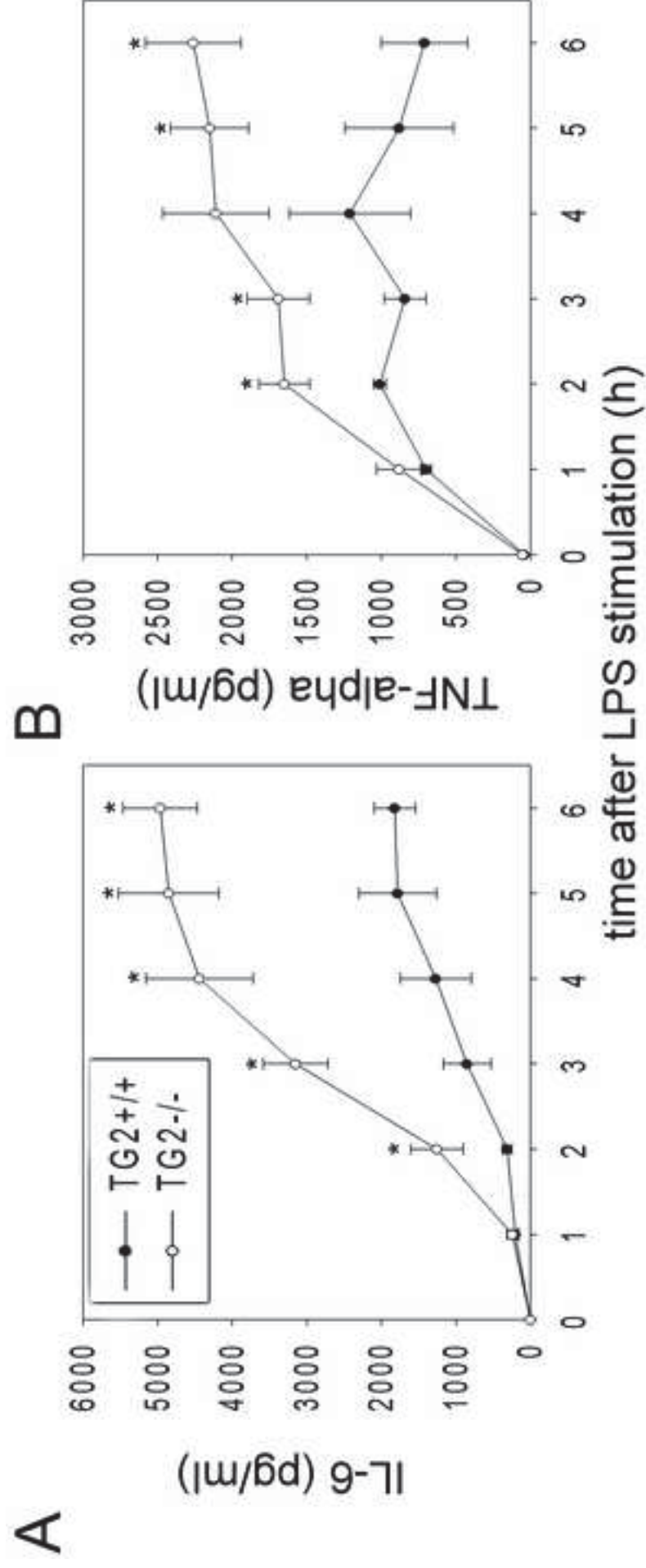


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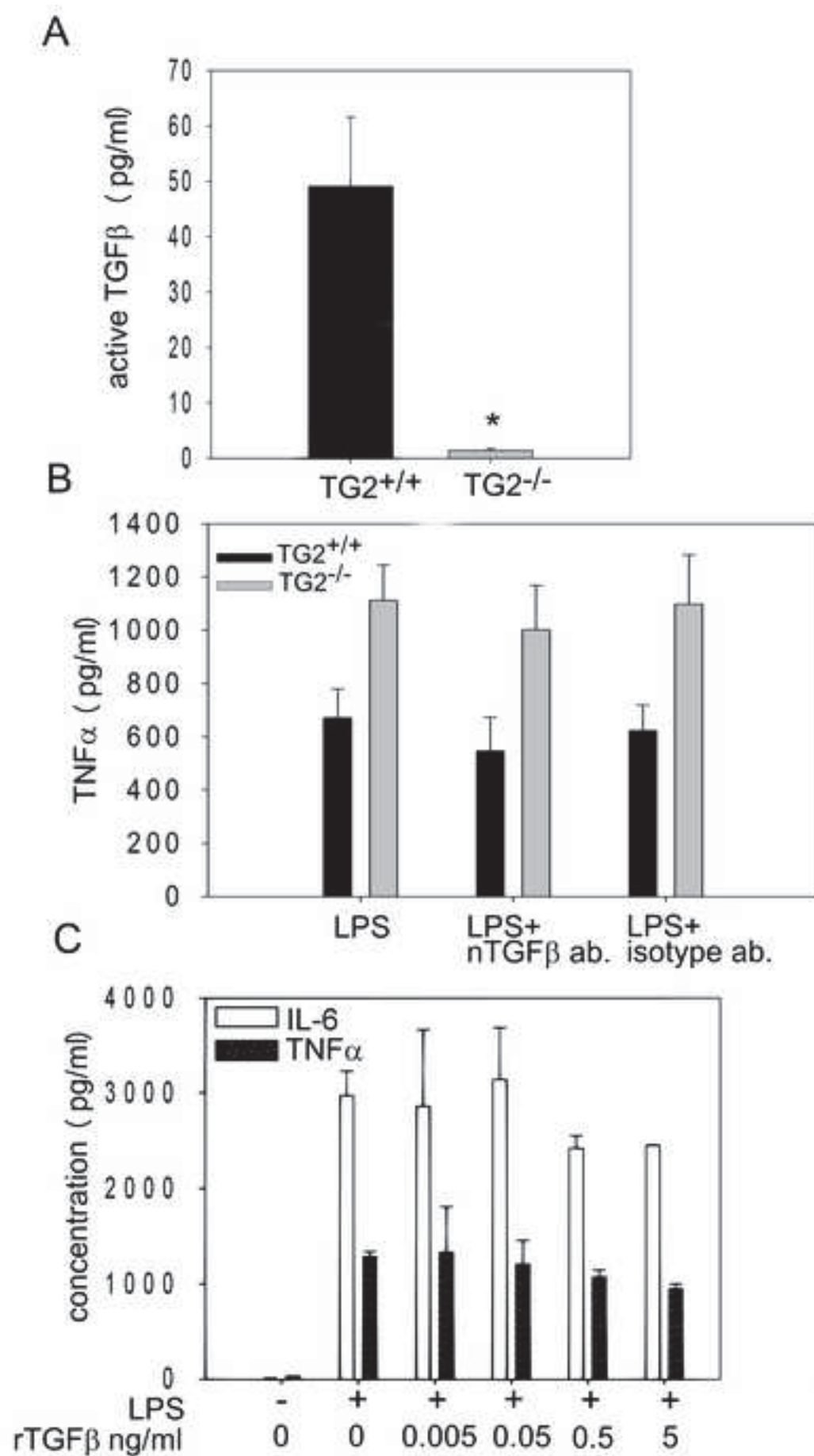
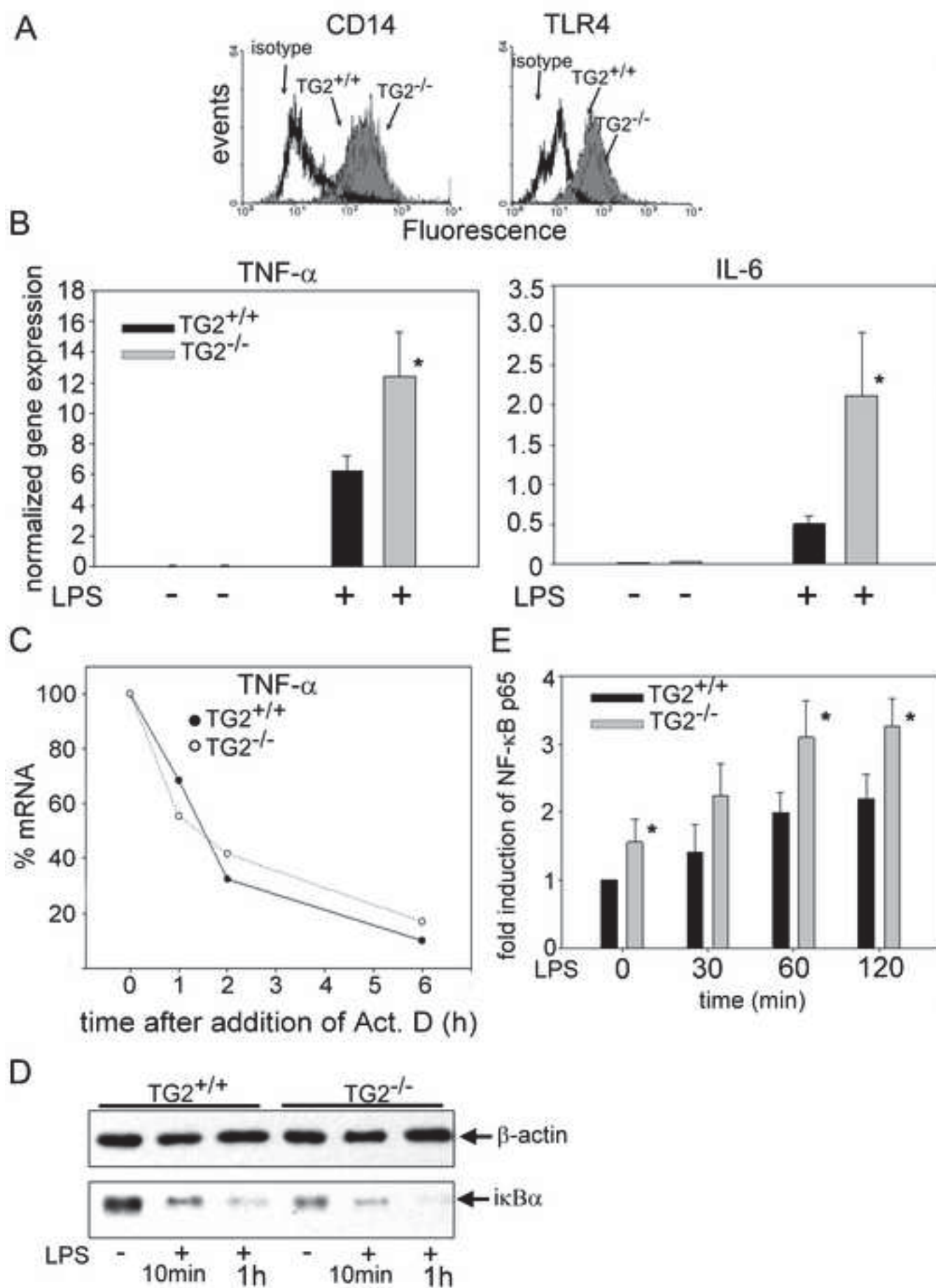




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